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The pluripotent cytokine pleiotrophin is induced by wounding in human mesangial cells

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Mesangial re-modeling and mesangial cell (MC) migration are features of several glomerular diseases including mesangiocapillary glomerulonephritis. *In vitro* investigations have recently identified ADAM-15, a multidomain adamalysin, as central to the migration of MC. The current study used array technology to investigate the expression of other genes in migrating cells and identified pleiotrophin (PTN), platelet-derived growth factor alpha polypeptide chain, colony stimulating factor, and four members of the tumor necrosis factor-α superfamily as major genes that were upregulated. Transcriptional induction of PTN was confirmed by reverse transcription-polymerase chain reaction and Northern blotting and induction of the protein by Western blotting and immunohistochemical localization. PTN was observed associated with mesangial 'hillocks' in confluent MC cultures. In contrast, in models of migration, migrating cells had the highest expression of cell-associated PTN. PTN protein was less evident, however, in the conditioned medium of MCs. Treatment of MC with heparanase removed PTN from the cells suggesting that its localization was owing to an association with heparan sulfates on the cell surface or in the extracellular matrix. This is the first description of the expression of PTN by human MCs and the data suggest that it is rapidly induced in cells that are triggered to migrate. The result of this induction is currently under investigation.

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KEYWORDS: cytokines; glomerulonephritis; renal injury; mesangial cells

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The mesangium, comprising mesangial cells (MCs) and the extracellular matrix that they synthesize, the mesangial matrix, forms the structural core of the glomerulus. In addition to this architectural function, however, the mesangial matrix has a central role in homeostasis and the normal functioning of the glomerulus. The major components of this matrix include collagen type IV, laminin, fibronectin, and a number of different heparan sulfate, chondroitin sulfate, and dermatan sulfate proteoglycans. In the normal kidney these components are maintained at constant levels owing to the balance between the synthesis and deposition of extracellular matrix components and their proteolytic breakdown. In a number of renal diseases this equilibrium is shifted and, as a result, the normal composition of the mesangial matrix is altered leading to glomerulosclerosis and loss of renal function. Furthermore, in a number of renal diseases, MCs become motile and migrate through the remodeled matrix into the pericapillary space.[1] This migration is mediated through interactions with a range of extracellular matrix molecules such as thrombospondin,[2] fibronectin, [3] and heparan sulfate glycosaminoglycans.[4]

Our recent studies into the mechanisms involved in MC motility have revealed that ADAM-15, a member of the adamalysin family of cell surface molecules, is strongly involved in MC migration. The potential involvement of soluble mediators as factors synthesized during MC migration, however, has not been addressed. In a recent report, Henger *et al.*[5] used gene expression fingerprinting technology to examine differential gene expression profiles in the kidneys of patients with progressive fibrosis. Of nine genes identified as having a role in progression, seven were also associated with inflammation. The two genes associated solely with fibrosis were the β4 integrin chain and the soluble mediator pleiotrophin (PTN).

PTN is a member of the midkine family of molecules and has over 50% homology with midkine. Both are retinoic acid-inducible molecules, although, unlike midkine, PTN has been shown to be induced by platelet-derived growth factor alpha polypeptide (PDGFA), and may be a downstream effector of this cytokine. PTN is a 136 amino acid, 15.3 kDa protein, and is also known as heparin binding growth-associated molecule, embryonic differentiation factor, and heparin affinity regulatory peptide. It is very highly conserved across species, with over 98% homology between human,

mouse, rat, and bovine.[6] PTN is involved in a range of biological processes; it has important functions in renal development[7,8] and induces proliferation of human blood peripheral mononuclear cells.[9] Elevated levels have also recently been shown in osteoarthritis.[10] As a proto-oncogene, it has been implicated in tumor angiogenesis, cell differentiation, and the development of spleen and lung cancers.[11,12] Furthermore serum PTN levels have been suggested as a marker for these diseases.

As PTN is upregulated in renal disease, the current study was undertaken to establish whether PTN was synthesized by MC and whether its expression was increased in MC during migration.

RESULTS

Wounded monolayers of human MCs upregulate PTN mRNA expression

Confluent MC were multi-scratched and messenger RNA (mRNA) was extracted. Complementary DNA was prepared using the cytokine-specific primers supplied with the common cytokine gene 'Super Array' kit. Scratched cultures were compared to corresponding unscratched control cultures. The expression of several cytokines was markedly upregulated following scratching, including the pluripotent cytokine PTN and the tumor necrosis factor (TNF) superfamily member, TNF (ligand) superfamily member (Figure 1).

The arrays were subjected to densitometric analysis and the ratio of each mRNA spot was normalized to that of the housekeeping genes on each respective blot before comparison between blots. The results are presented in a bar chart (Figure 2). This analysis confirmed the relative increase in the mRNA coding for PTN and also demonstrated upregulation of other genes, such as colony stimulating factor 1, and several members of the TNF super family. There were also genes, however, which showed reduced activity, including transforming growth factor beta 2. These results were confirmed by stripping the blots and (following confirmation of the total removal of probes) repeating the experiment using mRNA from a second MC culture with and without multi-scratch wounding as before (data not shown).

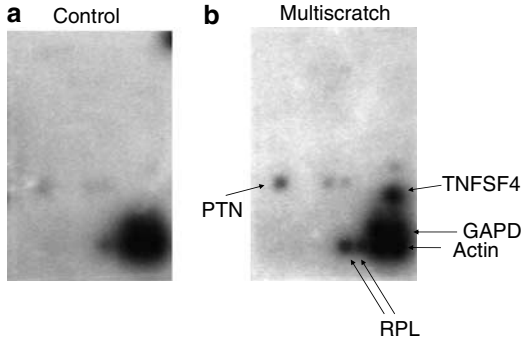

Figure 1 | A representative expression array of mRNA from control and multi-scratched MC cultures. mRNA was isolated after72 h incubation from (**a**) untreated cells or (**b**) following multi-scratch wounding. mRNA was labeled and hybridized to a common cytokine array as described. Two of the most strongly induced cytokines (PTN and tumor necrosis factor (ligand) superfamily member 4) are shown together with the three control, housekeeping genes (ribosomal protein L, glyceraldehyde-2-phosphate dehydrogenase, and actin B).

In order to confirm the findings from the gene array experiments, primers were designed from the sequences of PTN deposited in the GenBank database. There were several distinct sequences deposited that are reported to code for PTN but all had an overlapping consensus sequence. Primers were designed within this consensus sequence and, using reverse transcription-polymerase chain reaction (RT-PCR), these confirmed the presence of the PTN mRNA in MCs and its time-dependent induction, which peaked at 3 h after cells were subjected to multi-scratch wounding. The levels of mRNA were found to be somewhat variable between MC cultures, but the PTN:actin ratio was consistently found to be increased in the migrating cells relative to the non-migrating cells (Figure 3a and b). In addition, Northern blotting was carried out using RNA prepared from scratched and control MC cultures and these investigations confirmed both the presence of PTN mRNA and its induction in wounded cell monolayers (Figure 3c).

Wounded monolayers of human MCs upregulate PTN protein expression

Western blotting of the MC culture medium showed the presence of PTN, which was detected as a single band of 18 kDa. This band, however, was variably present and in some experiments PTN could not be detected at all in the culture medium (not shown).

Immunostaining MC with anti-PTN antibodies demonstrated punctate cell-surface staining for PTN. There was a marked increase in staining associated with 'hillocks' of MCs (Figure 4b). Furthermore, cell surface staining for PTN (Figure 4c) was reduced following heparitinase treatment (Figure 4d) confirming that PTN binding was mediated through an interaction with cell- and matrix-associated heparan sulfate.

In addition, MCs were shown to stain positive for PTN when analyzed by fluorescence-activated sell sorter (FACS). The amount of staining was increased when the cells were migrating following multi-scratch wounding. Figure 5 shows the shift of PTN staining at 72 h compared to non-migrating cells. This induction of cell-associated PTN was seen as early at 4 h and was present for at least 72 h. There was a time-dependent induction of cell-associated PTN beginning at 4 h (25% of cells in region M2 compared to 12% of control cells) following scratch wounding. This expression reached a plateau by 24 h and by 72 h 28% of the cells were in region M2 (Figure 5). Together these findings suggest that there is a mechanism for maintaining PTN in association with the cell and that there may also be a mechanism for triggering its release.

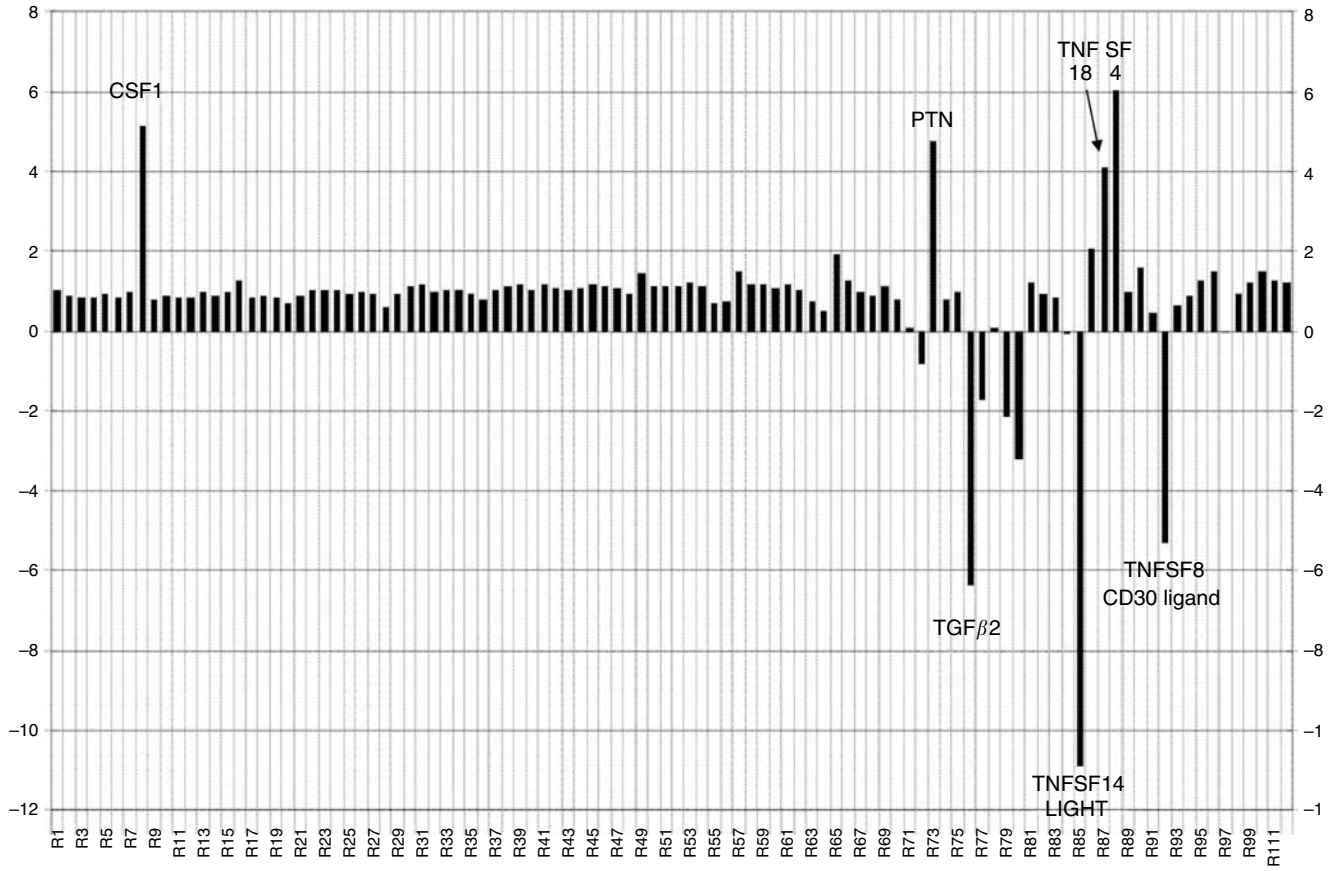

Figure 2 | Bar chart showing relative amounts of cytokine mRNA in migrating compared to non-migrating MCs. Cytokines showing the greatest induction or inhibition following scratching are labeled. Results are representative of two independent experiments.

Association between PTN and PDGFA chain expression

Platelet-derived growth factor (PDGF) is a strong mitogen and inducer of migration in MCs. Following multi-scratch wounding, PDGFA mRNA was induced in a time-dependent manner (Figure 6). To investigate the possibility that there was a causal link between the upregulation of PTN and that of PDGFA, cells were treated with either heparitinase or the PDGF receptor signaling blocker, AG1296, to inhibit PTN- or PDGF-dependent responses, respectively. Semiquantitative RT-PCR was then carried out to examine the subsequent effect on PDGF or PTN mRNA levels, respectively. Blocking MC responses to PDGF resulted in significant inhibition of the induction of PTN mRNA (Table 1A) at both 1 h (76.9% inhibition) and 3 h (59.8% inhibition), suggesting that PDGF may be a major factor involved in the stimulation of PTN release in MC. Importantly, however, heparitinase treatment significantly inhibited the induction of PDGFA mRNA by 81.8% at 1 h and 78.7% at 3 h (Table 1B), suggesting an amplification loop involving the two cytokines.

DISCUSSION

We have recently begun *in vitro* investigations into the mechanisms involved in initiating and controlling MC migration and in the current study we have addressed the possibility that there are soluble mediators released when MC are induced to migrate. This work is the first report that PTN is produced by MCs *in vitro*, and furthermore that PTN was induced in migrating cells as shown by cytokine array. In this migration assay, following the initial period of migration the cells will begin to proliferate as they expand – as in the typical reaction of cells during the wound healing process. PTN binds to extracellular matrix and with high affinity to heparin.[13] Thus treatment of cells with heparitinase removes bound PTN and in our study this also reduced the induction of PDGFA chain mRNA, suggesting that PTN (but we cannot rule out other additional heparin-binding growth factors) directly controls synthesis of PDGF by MC. PTN, colony stimulating factor, and members of the TNF superfamily were all increased with PDGFA chain by the induction of migration. PDGF has previously been implicated in the production of PTN,[14] and so the presence of the mRNA for this molecule in our arrays may indicate the specific stimulation of a small group of cytokines that interact and are possibly co-regulated in migrating cells. This is further supported by our results using blockade of the PDGF-receptor signaling cascade by AG1296. This prevented the induction of PTN mRNA, confirming an association in controlling MC function and a potentially important

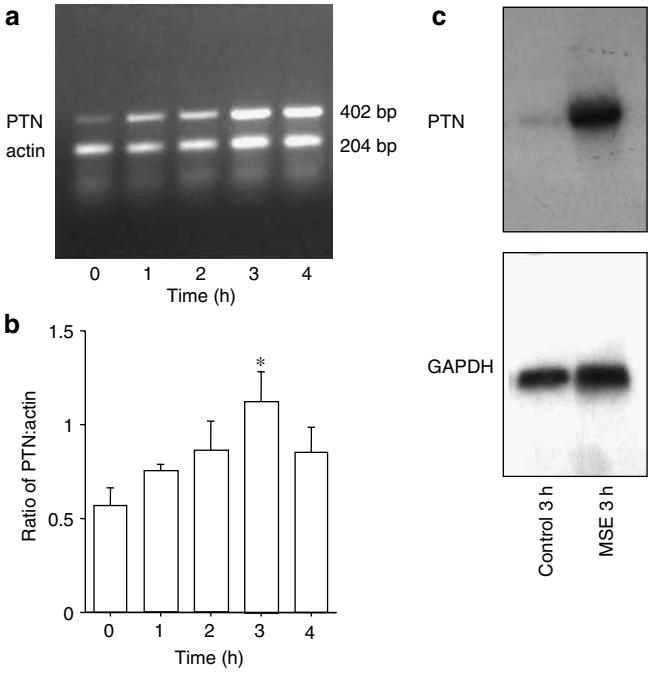

Figure 3 | Time course of induction of PTN. (**a**) Multi-scratch wounds were inflicted on human MC and RNA was extracted at the indicated times. The RNA was amplified by RT-PCR and products separated in 2% agarose gels with actin as the housekeeping gene. (**b**) Results shown in are the means $\pm$s.d. of the densitometric analyses from seven independent experiments. (**c**) In addition, the mRNA from 3 h incubations was compared by Northern blotting to that prepared from control non-migrating cells by hybridizing with a PTN-specific probe. Equal loading was confirmed by stripping the blot and re-probing for glyceraldehyde-2-phosphate dehydrogenase (*$P<0.05$ compared to 0 h).

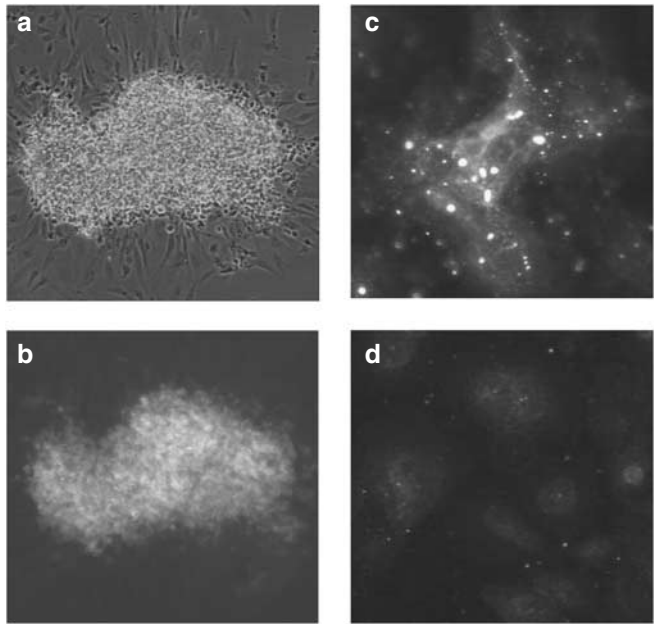

Figure 4 | PTN is expressed on the surface of MC. Immunohistochemical staining of human MCs for the PTN protein demonstrated increased amounts associated with MC hillocks. Mesangial cells were grown to confluence and allowed to produce the distinctive protein rich 'hillocks'. Cells and hillocks were viewed under brightfield (**a**) or stained with anti-PTN and visualized with fluorescein isothiocyanate conjugated second antibody (**b**–**d**). In (**c** and **d**), cell surface staining for PTN is shown reduced following heparitinase treatment. Mesangial cells were grown to confluence as before and incubated with either PBS (no treatment) as control (i) or heparitinase (ii) following which immunohistochemistry was performed as before in order to visualize the presence of PTN on the cells.

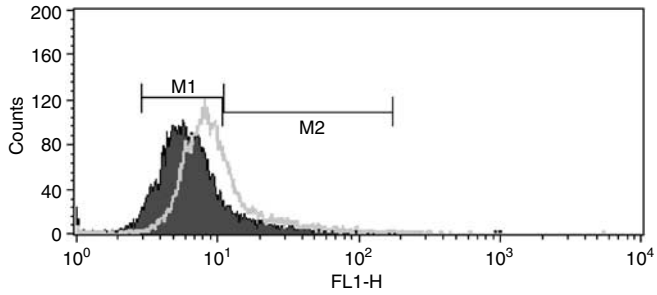

Figure 5 | FACS analysis of PTN expression by human MCs. Cells were subjected to multi-scratch wounding or left untreated and prepared for FACS analysis of PTN expression as described in Materials and Methods. Results show the increased expression of PTN at 72 h and are representative of three independent experiments. Solid peak shows the PTN expression in non-migrating cells. Gray line represents the shift in PTN expression in migrating cells.

interdependence of these two cytokines. PTN has recently been shown to also bind specifically to a chondroitin sulfate proteoglycan, with a downstream signaling system which includes extracellular signal-regulated kinase and phosphatidylinositol 3 kinase.[15] In our studies, however, we found no effect of digesting cell surface chondroitin sulfate chains with chondroitinase ABC (not shown).

There are several reports of the PTN molecule being upregulated in cancers. Initial reports described the upregulation this cytokine in breast[16] and spleen cancer,[11] although several other types of cancer are now believed to show an increased production of this molecule, for example, pancreatic cancer,[17] and lung cancer[12] and testicular cancer.[18] Pleitrophin has also been implicated in both rheumatoid arthritis[19] and osteo-arthritis.[10]

Although considered primarily as a mitogen, there has been one report describing PTN involvement in wound healing in rat dermal cells.[13] This is one of the first reports demonstrating the involvement of PTN in the migration of cells *in vitro*, and is indicative of the multi-functional but related role possessed by this growth factor. There has, however, been a recent report describing the involvement of PTN in osteoprogenitor cell migration.[20] In addition, PTN and its receptor tyrosine phosphatase-zeta have been implicated in regulating glioblastoma cell motility both in the soluble phase[21] and when PTN was immobilized.[22] There is also, however, a contrasting report where PTN was found to have no effect on either glioblastoma or endothelial cell migration.[23]

All of the reports referred to above associate the molecule with a role in angiogenesis or mitogenesis or in the migration

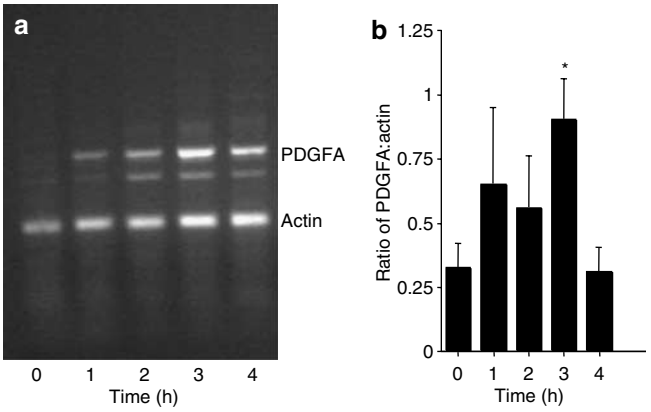

Figure 6 | PDGFA chain is induced in scratch-wounded MC. RT-PCR for PDGFA demonstrated a time-dependent increase in the PDGFA chain mRNA in migrating human MCs. Multi-scratch wounds were inflicted on human MC and was RNA extracted at the indicated times. PDGF mRNA was then amplified by RT-PCR and products separated in 2% agarose gels with actin as housekeeping gene. (**a**) The gel is representative of five separate experiments and (**b**) the mean densitometry values (±s.d.) of PDGFA corrected for the density of the corresponding actin values are shown.

Table 1 | (A) Semiquantitative RT-PCR for PTN in AG 1296-treated cells[a] and (B) semiquantitative RT-PCR for PDGFA chain in heparitinase-treated cells[a]

(A)

Time from scratch induction	1 h	3 h
Without treatment	1.49±0.7	1.13±0.21
With treatment	0.35±0.17	0.60±0.25

(B)

Time from scratch induction	1 h	3 h
Without treatment	4.95±0.9	3.75±0.8
With treatment	0.90±0.18	0.80±0.20

PDGFA, platelet-derived growth factor alpha polypeptide; PTN, pleiotrophin; RT-PCR, reverse transcription-polymerase chain reaction.
[a]MC cultures were multi-scratched and RNA extracted as described in the Materials and Methods section.
The densitometry ratio of either PTN (A) or PDGFA (B) to that of the housekeeping gene (β-actin) were calculated and the results are expressed as the mean of these±s.d. of three independent experiments.

of cells, often in cancer or inflammation. These results would tie in with our finding of the involvement of the cytokine in our *in vitro* model of wound healing, and provide a new possible target for the modulation of healing wounds *in vivo*.

In conclusion, PTN mRNA was detected by using cytokine arrays and was shown to be induced when MC were stimulated to migrate. We have confirmed that these cells *in vitro* both express the PTN mRNA and produce the PTN protein. Having defined a role for PTN *in vitro*, studies *in vivo* will now be possible to define its involvement as a paracrine or autocrine factor. Given the potency of this molecule it is likely that its generation *in vivo* would have a marked effect on glomerular function.

MATERIALS AND METHODS

All general reagents were from Sigma-Aldrich, Poole, Dorset, UK), unless stated otherwise. All tissue culture reagents were obtained from BRL Life Technologies (Gibco BRL), Gaithersburg, MD, USA. Electrophoresis reagents were purchased from BioRad Laboratories Ltd, (Hemel Hempstead, Herts, UK).

Cell culture and identification

MC were cultured from human glomeruli obtained by the serial sieving of the kidney. MCs were maintained in Rosewell Park Memorial Institute media-1640 containing 20% v/v fetal calf serum. Before experimental procedures MC were growth arrested for 48 h by culture in medium containing 0.2% (w/v) lactalbumin hydrolysate (Difco Laboratories, Detroit, MI, USA) in the absence of serum.[24] The cells were confirmed to be MCs by morphology and the use of immunohistochemistry as described.[25] Cells showed positive staining for intracellular myosin fibrils, and were negative for factor VIII and cytokeratin. Three separate cell lines were used for the RT-PCR and Western blot analysis, two of which were used in the cytokine array study.

Confluent MC were multi-scratch wounded in order to induce a high percentage of migrating cells in the cultures.[26] RNA was collected from both scratched and control dishes using RNA Isolator (Genosys Ltd, Cambridge, UK) according to the manufacturers instructions.

Treatment with heparatinase

Semiconfluent cell layers grown in 8 well chamber slides were scratch wounded and incubated for a further 24 h. To remove cell-surface heparan sulfate glycosaminoglycan chains they were then washed with phosphate-buffered saline (PBS) and incubated with 0.3 mU/ml each of heparatinase I, II, and III in the absence of serum for 30 min. The cultures were then fixed and stained for PTN as detailed below.

Common cytokine super array analysis

Complementary DNA was prepared using the cytokine-specific primers supplied with the common cytokine gene 'Super Array' kit.[27] The probes were prepared using the 'Strip Easy' protocol and materials[28] in order to allow the membranes to be used for three separate hybridizations. Radiolabeled complementary DNA transcripts were hybridized to the two identical 'Super Array'[27] membranes pre-spotted with sequences specific to each of a variety of common cytokines. Cytokine spot densitometry was quantified by use of the 'Chemi Doc Gel Quantification System,' with 'Quantity One' Software (BioRad Labs), and data analyses performed using the 'super array' GEArray Analyzer software. The membranes were then stripped using the 'Strip Easy' manufacturers protocol and the removal of the probes was confirmed. An unconnected experiment was then performed in order to ensure a different pattern of results was obtained using the membranes, which were then re-stripped and the multi-scratch experiment repeated as before using a different MC line.

RT-PCR

Confluent 35 mm dishes of MCs were growth arrested and multi scratches introduced as described. Total RNA was extracted using RNA Isolator at various time points following the initiation on the scratch and complementary DNA was prepared by the reverse transcription of 1 μg of RNA using random primers and the

equivalent of 0.05 μg was amplified by PCR using the following primers specific for PTN and actin and PDGF A. (Genosys Ltd, Cambridge, UK).

PTN primers:
5′-CCTTCTTGGCATTCATTTTCAYACTGG-3′
5′-GAGGTTTGGGCTTGGTCAGTTTGC-3′
Cycle number: 35
Actin primers:
5′-GGAGCAATGATCTTGATCTT-3′
5′-CCTTCCTGGGCATGGAGTCCT-3′
Cycle number: 30
PDGF A primers:
5′-AATTTCGCCGCCACAGGAGA-3′
5′-ACGGGGGCCAGATCAGGAAG-3′
Cycle number: 35

Northern Blot analysis

Total RNA (up to 10 μg) was run on a denaturing agarose gel and transferred by vacuum blotting onto a Hybond nylon membrane (Amersham, UK). mRNA for PTN was detected by hybridization with a ^{32}P-labeled probe prepared from PCR product and detected as described previously.[29]

Western blotting

MC-conditioned medium was collected, and an equivalent volume, corrected for cell number as determined by the reduction of Alamar Blue,[30] was applied to 7.5% sodium dodecyl sulfate-polymerase chain reaction gels and separated under reducing conditions. Following electrophoretic transfer to nitrocellulose and blocking in 5% skimmed milk in PBS containing 0.5% Tween 20, the blots were incubated with 0.1 μg/ml anti-PTN antibody (R&D systems, Abingdon, Oxon, UK) in PBS, 0.1% Tween 20, 1% bovine serum albumin overnight. The blots were then incubated with horseradish peroxidase-conjugated anti-Goat IgG for 2 h, and antibody binding was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK). Detected bands were quantified by use of the 'Chemi Doc Gel Quantification System,' using 'Quantity One' Software (BioRad Labs).

FACS Analysis of cell-associated PTN

Following multi-scratch wounding for the appropriate times, cells were removed by mild trypsin/ethylenediaminetetraacetic acid treatment. The trypsin was neutralized by the addition of 10% fetal calf serum and the cells pelleted by centrifugation (1500 r.p.m. 7 min). They were then fixed and permeabilized in 100 μl of Cytofix/Cytoperm for 20 min and washed $\times 2$ in Perm/wash solution (BD Biosciences 10 975 Torreyana Road San Diego, CA, USA), according the to the manufacturers protocol. Cells were incubated with goat anti-PTN antibody diluted to 1/500 at 4°C for 30 min. After washing $\times 2$ in Perm/wash solution cells were incubated with anti-goat fluorescein isothiocyanate (1/400) for 30 min at 4°C, washed and finally resuspended in 250 μl of Perm/wash solution and 10 000 cells were analyzed per sample by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK).

Immunohistochemistry

MCs were grown to confluence in eight-well chamber slides, and half the cells were removed by scratching. The cells were incubated for a further 24 h at which point the cells were fixed in ice cold acetone:methanol (50:50) for 5 min, washed, and incubated with anti-PTN antibody 1/200 in PBS bovine serum albumin (1%) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, sc-1394: Autogen Bioclear, Holly Ditch Farm, Mile Elm, Calne, Wiltshire SN11 0PY) for 3 h at room temperature. The cells were then incubated for a further 3 h in the presence of anti-goat fluorescein isothiocyanate conjugated secondary antibody (Sigma-Aldrich, Poole, Dorset, UK) diluted 1/80, washed, mounted in Vectashield (Vector Laboratories Burlingame, CA, USA) and visualized using a fluorescence microscope (Leitz Dialux 20 EB).

Inhibition of PTN and PDGFA transcription

To investigate whether there was a causal relationship between the upregulation of PTN and PDGFA, cells were incubated with AG1296, an inhibitor of PDGFA signaling, or heparitinase, to disrupt presentation to the PTN receptor. Semi confluent cell layers grown in eight-well chamber slides, or 24-well plates were washed with PBS and then further incubated in the presence of 3.8 μM AG1296 for up to 6 h, and RNA collected for RT-PCR for PTN or for 24 h at which point the cells were washed fixed and stained as above.

For inhibition of PTN effects, semiconfluent cell layers grown in 24 well plates were washed with PBS and then further incubated in the presence of heparatinase I, II, and III (0.3 mU/ml) for up to 6 h. The RNA was collected for RT-PCR for PDGFA as detailed above.

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